Investigating the roles of *NDJ1* and *TID1* in crossover assurance in
Saccharomyces cerevisiae

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TITLE: Investigating the roles of *NDJ1* and *TID1* in crossover assurance in *Saccharomyces cerevisiae*

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ABSTRACT

Investigating the roles of *NDJ1* and *TID1* in crossover assurance in *Saccharomyces cerevisiae*

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Meiosis is the specialized process of cell division utilized during gametogenesis in all sexually reproducing eukaryotes, which consists of one round of DNA replication followed by two rounds of chromosome segregation and results in four haploid cells. Crossovers between homologous chromosomes promote proper alignment and segregation of chromosomes during meiosis.

Crossover interference is a genetic phenomenon in which crossovers are non-randomly placed along chromosomes. Crossover assurance ensures that every homologous chromosome pair obtains at least one crossover during Prophase I. Crossovers physically connect homologous pairs, allowing spindle fibers to attach and separate homologs properly. However, some organisms have shown an ability to segregate chromosomes that fail to receive at least one crossover, a phenomenon termed distributive disjunction.

In *Saccharomyces cerevisiae*, mutation of either *Tid1* or *Ndj1* results in a similar defect in crossover interference. The overall number of crossovers is not substantially different from the wild type, however they are distributed more randomly with respect to each other. In this thesis, the roles of *Tid1* and *Ndj1* on crossover assurance and distributive disjunction have been further elucidated through use of knock-out mutants and tetrad dissection.

To analyze meiotic chromosome segregation in isogenic *tid1* and *ndj1* strains, the spore viability of dissected tetrads was utilized as an indirect measure of nondisjunction events. An elevated number of 2- and 0- spore viable tetrads were seen in *ndj1*, but not *tid1* yeast, confirming previous results. Elevated 2- and 0- spore viable tetrads are an indication of meiosis I (MI) nondisjunction, commonly resulting from failure of crossover formation. These results suggest crossover assurance is disrupted in *ndj1*, but not *tid1* mutants. However, MI chromosome segregation is an indirect readout of crossover formation; distributive disjunction, for example, can lead to proper segregation of achiasmate chromosomes.

To determine if distributive disjunction is functional in yeast, wild type, *tid1* and *ndj1* versions of diploid yeast carrying a single homeologous pair of chromosomes were constructed. These strains have one chromosome (chr. III or V) replaced with one from a closely related species of yeast. The homeologous chromosome functionally replaces the homolog, however crossovers are significantly reduced between homeologs. A spore viability pattern typical of MI nondisjunction was detected in *ndj1* mutants, but not in *tid1* mutants. In the context of these homeologs, this pattern is suggestive of a role for *Ndj1*, but not *Tid1*, in distributive disjunction. Further, these results suggest that *tid1* and *ndj1* mutant yeast may not be different in their competence for crossover assurance.

To directly assay competence for crossover assurance in native mutants, the incidence of E_0 chromosome pairs (those lacking crossovers) was determined. To do this we assayed crossover formation along the length of chromosome III of isogenic wild type, *ndj1* and *tid1* mutant strains. The incidence of E_0 chromosomes was comparably elevated in both *tid1* and *ndj1* mutant yeast, suggesting that crossover assurance is nonfunctional in both strains.

We find evidence that supports the idea that interference and assurance are genetically linked. Our data also suggests that distributive disjunction may be genetically separable from some meiotic genes.

Keywords: meiosis, crossover assurance, nondisjunction, Ndj1, Tid1, yeast.

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Introduction

Life cycles: Mitosis and Meiosis

In the somatic cell cycle, cells live most of their lives in a vegetative, G_1 phase. When ready to duplicate, they initiate the process of mitosis. This begins with duplication of chromosomes in S phase, producing two identical DNA molecules connected at a centromere. These pairs are called sister chromatids and together constitute one chromosome. Sister chromatids are separated into two cells during mitosis (M phase) producing two daughter cells identical to the original parent cell. All single celled organisms, and somatic cells of multicellular organisms undergo this process of mitosis to propagate their DNA and “grow” or duplicate.

Most eukaryotic organisms have a sexual life cycle in which genetic information from two parents are combined to form offspring. The sexual life cycle generally requires that two haploid gametes fuse to form a diploid zygote (Figure 1). Haploid gametes are produced in a process called gametogenesis. Meiosis is the specialized process of cell division in gametogenesis that consists of one round of DNA replication followed by two rounds of chromosome segregation. A complete meiotic division produces gametes containing half the chromosomal complement of the parent cell. Diploid ($2n$) organisms have two copies of each chromosome, one set from mom and one set from dad. The pairs of chromosomes are called homologous chromosomes and are identical in gene order, but prone to allelic variation. Meiosis produces four haploid ($1n$) cells (each with a single copy of each homolog) from one diploid cell. Meiosis is highly conserved among eukaryotic organisms, including humans.

Figure 1. Diagram of the sexual lifecycle of a diploid eukaryote. Egg and Sperm are used to show the difference in “male” and “female” meiotic products. Not all eukaryotes have physical differences between mating types, diploid and haploid life forms. *Saccharomyces cerevisiae* is an example of an organism that is visually identical in all life stages (not including mitosis and meiosis).

Figure 2. Comparison of mitotic and meiotic chromosome segregation. (Keckner 1996).

Most of the sexual cell cycle is similar to the asexual cell cycle, in that there are G_1 , S , and G_2 phases. The differences lie in the M phase. In mitosis, there is one round of DNA segregation, where sister chromatids separate from each other to produce two identical daughter cells. In meiosis, there are two rounds of chromosome segregation: Meiosis I, also known as the reductional division, separates homologous chromosomes and Meiosis II, also known as the equational division, separates sister chromatids (Figure 2). Meiosis I is further divided into prophase, metaphase, anaphase, and telophase. Prophase I is characterized pairing and crossing over between homologous chromosomes. Connected homologous chromosomes are aligned in

Metaphase I, and separated to opposite poles during Anaphase I. Chromosomes are then decondensed during Telophase I, followed by cleavage of the two daughter cells during cytokinesis (Figure 3). During meiosis II sister chromatids separate without the replication step, resulting in formation of the final four haploid germ cells. Meiosis II is very similar to mitosis, in that sister chromatids are separated, and no crossing over takes place.

Figure 3. General stages of Meiosis. See text for description

Prophase I is the longest phase of meiosis and is broken up into 5 stages: Leptotene, zygotene, pachytene, diplotene, and diakinesis (Figure 4). During leptotene, chromosomes begin to condense to form visible strands within the nucleus. The axial elements of the synaptonemal complex begin to form and double strand breaks (DSBs) begin to appear in DNA. In the leptotene/zygotene transition, the telomeres cluster at a region of the nuclear envelope near the

yeast centrosome, forming bouquet-like configurations (Figure10). During zygotene, homologs continue to condense and synapse. Pachytene is marked by full synapsis, completed by the assembly of the central element, and crossing over. Once synapsis is completed, each homologous pair is fully aligned and connected by the synaptonemal complex. Nonsister chromatids of homologous chromosomes exchange genetic material through crossing over and form structures called chiasmata. Centromeres of homologous chromosomes begin to separate during diplotene, but the two homologs remain attached at chiasmata. In diakinesis, chromosome condensation continues, and towards the end of prophase I the nuclear envelope breaks down and the spindle fibers form.

Figure 4. Stages of Prophase I. See text for description (Page and Hawley 2003).

Nondisjunction and Aneuploidy

Crossovers and the resulting chiasmata are essential for the proper segregation of chromosomes during meiosis I. In the absence of crossover formation, there is no connection between the chromosomes, resulting in a lack of tension between the spindle poles during meiosis. The main system cells use to ensure proper segregation fails in the absence of this tension, frequently resulting in the mis-segregation of chromosomes, or nondisjunction.

Aneuploidy is a condition in which a cell has an incorrect number of chromosomes. Figure 5 illustrates one possible outcome of nondisjunction compared to disjunction (normal chromosome segregation). Aneuploidy resulting from nondisjunction is a problem because of the sexual life cycle. For example, in humans, each germ cell is a potential half of a human being, and if the sperm or egg of a zygote were aneuploid, the result would be an embryo with an incorrect number of chromosomes. The most common human diseases resulting from aneuploidy are Down syndrome (trisomy 21) and sex chromosome trisomies (XXX, XXY, XYY). In humans, the incidence of aneuploidy is fairly high: about 0.3% of live born newborns, about 4% of still births and about 35% of clinically recognized spontaneous abortions (Hassold and Hunt 2001). The same study found a higher rate of nondisjunction in female oogenesis compared to male spermatogenesis for all aneuploid gametes.

Figure 5 Diagram of disjunction and nondisjunction. This figure shows one homologous pair of chromosomes, assuming all other chromosomes segregate normally. In haploid yeast, some types of aneuploidy can result in viable spores: an extra copy of a chromosome results in a viable cell, while if an entire chromosome is missing, the cell will not be viable.

Crossover formation

To properly halve chromosome number in meiosis, homologous chromosomes must disjoin properly at the first meiotic division. The crossovers formed during Prophase I of meiosis play a large role in ensuring proper segregation of the homologs. Crossovers establish chromatin bridges (chiasmata) between homologs that ensure proper orientation of homologs to opposite poles along the Meiosis I spindle (Page and Hawley, 2003). This alignment facilitates proper disjunction during meiosis. In order for crossovers to form, the DNA must be broken. The following sections describe the process of crossover formation beginning with the formation of breaks and ending with their repair and chiasmata formation.

Double Strand Breaks

Recombination is initiated by meiosis-specific double strand breaks (DSB) (See Figure 7). Double strand breaks are most commonly made in hotspots, or regions that have high rates of recombination. However, cellular factors other than primary DNA sequence play a role in determination of DSB sites. Studies have shown that chromatin structure plays a role in determining where DSBs occur, and that these effects can function several thousand nucleotides away (Wu and Lichten 1995).

A protein called Spo11 makes targeted DSBs in both strands of one chromatid in the homologous pair (Keeney et al. 1997). Spo11 is part of a protein complex that (in *S. cerevisiae*) involves products of nine genes. The meiosis specific genes are *SPO11* (Cao et al. 1990), *MEI4* (Menees et al. 1992), *MER2* (Rockmill et al. 1995), and *REC102*, *REC104* and *REC 114* (Bullard et al. 1996). The genes that also function in repair of DSBs in non-meiotic cells are *RAD50* (Alani et al. 1990; Sugawara and Haber 1992; Schiestl et al., 1994), *MRE11* (Ajimura et al. 1992; Johzuka and Ogawa 1995), and *XRS2* (Ivanov et al. 1992, 1994).

Synaptonemal complex

Homologous chromosomes synapse with each other to form the synaptonemal complex (SC) during meiotic prophase I. The SC is a zipper-like protein structure that is assembled between homologous chromosomes during prophase I of meiosis. Each SC is a tripartite structure that has two dense parallel structures called lateral elements that are separated by a central element (Figure 6). Each lateral element represents the protein backbone of a pair of sister chromatids: most of the chromatin is located outside of the complex, folded into a series of loops that are attached at the lateral element. This protein backbone is called the axial element until its incorporation into the SC. In normal meiosis, all chromosomes undergo full synapsis. In humans, the sex chromosomes are physically very different but still have some homologous regions and undergo partial synapsis (full synapsis on the Y chromosome with part of the X chromosome). Recent evidence suggests that a smaller chromosome will “stretch” in order to synapse with its longer homolog, and/or the larger chromosome will condense further to allow for more synapsis, a process called synaptonemal adjustment (Henzel et al. 2011).

The formation of the SC is correlated to condensation, pairing, recombination and disjunction of homologous chromosomes. The lateral elements begin to form in leptotene and the completion of the SC marks the beginning of pachytene (Figure 4). However, it is now evident that the SC is not required for genetic recombination. Some mutant yeast cells unable to form the SC can still recombine (Sym and Roeder 1994). The SC is now thought to function as a scaffold to allow interacting chromatids to properly form chiasmata. The SC must also disassemble during diplotene/diakinesis for proper disjunction of homologous chromosomes.

Figure 6. Synaptonemal Complex. The SC is a proteinaceous structure formed by a lateral element (LE) and central element (CE). The LE is comprised of cohesins (Rec8 for example), structural proteins, and the HORMA-domain proteins Hop1 and Red1. The CE is comprised of transverse filaments formed by proteins including Zip1 (Castro and Lorca 2005).

Double Strand Break Repair

Crossover formation is initiated by the formation of DSBs. These DSBs are repaired using the homolog as a template and can either result in a crossover or a non-crossover. The double Holliday junction pathway generally results in a crossover and the synthesis-dependent strand-annealing pathway generally results in a non-crossover (Figure 7). Repair begins with resection of the 5' ends, and strand invasion of the homolog by the 3' overhang. After DSBs are formed, the MRX complex binds to the DNA on either side of the break. The Sae2 protein resects the 5' ends, creating 3' single stranded overhangs. The Sgs1 helicase “unzips” the DNA and Exo1 and Dna2 nucleases cut the single-stranded DNA to continue the 5'-3' resection. Replication protein A (RPA) then binds to the 3' overhangs (Wold 1997), and Dmc1 forms a filament of nucleic acid and protein on the RPA coated single stranded DNA. The single stranded overhang then invades the homologous chromosome, forming a displacement loop (D-loop). How the D-loop is resolved determines whether a crossover or non-crossover occurs.

In the double Holliday junction pathway, a DNA polymerase extends the end of the invading 3' strand using the invaded homolog as a template, forming a Holliday junction. The 3' overhang that is not involved in strand invasion also forms a Holliday junction with the homologous chromosome, forming a cross-like structure known as a double Holliday junction (Figure 7). The double Holliday junctions can then be resolved as crossover or non-crossover by nicking endonucleases. If one Holliday junction is cut on the crossing strand and the other is cut on the non-crossing strand, a crossover forms (one vertical cut on one Holliday junction and one horizontal cut on the other in Figure 7). Alternatively, if both Holliday junctions are cut on the crossing strands, a non-crossover will be produced (both horizontal cuts in Figure 7).

In the synthesis-dependent strand-annealing (SDSA) pathway, the invading single stranded DNA serves as a primer for DNA polymerization, using the invaded strand as a template. The main difference from the dHJ pathway is that no Holliday junction occurs between any of the strands. As DNA synthesis proceeds, branch migration can displace the newly synthesized strand back to pairing with its original sister chromatid partner. This generates a gene conversion event, where only one chromatid obtains a new DNA sequence (Figure 7).

Roughly 30% of double strand breaks are repaired to form crossovers, while the remaining are repaired as non-crossovers. Several lines of evidence suggest that the crossover/non-crossover decision is regulated. The regulation of this decision point is discussed in the following sections.

Figure 7. DSB repair pathways.

Crossover control

Crossovers are required to create tension between spindle poles and facilitate the proper segregation of homologs. Eukaryotes have evolved several controls to ensure proper crossover placement. There are two known mechanisms that regulate crossover formation in most eukaryotes. Crossover assurance is the mechanism that guarantees the formation of at least one crossover between every homologous chromosome pair during Prophase I. Crossover interference is another mechanism that ensures non-random placement of crossovers along chromosomes (Hillers 2004). Many studies have shown that mutations that affect one mechanism also affect the other, suggesting that the two mechanisms are results of the same controls (Jones and Franklin 2006).

Crossover assurance

Crossover assurance describes the observation that each homolog pair typically receives at least one crossover, termed the obligate crossover. The incidence of bivalents lacking a crossover is lower than expected based on the total number of crossovers per bivalent. For example, homologs in *C. elegans* receive an average of one crossover per chromosome pair, yet each chromosome still obtains the obligate crossover. Figure 8 shows crossover distribution under random placement (Figure 8A) and when crossover assurance is functional (Figure 8B).

One group made the observation that crossovers seem to be maintained at the expense of non-crossovers, a phenomenon termed crossover homeostasis. Martini et al. (2006) showed that when the number of DSB events is decreased, with an allelic series of *spo11* mutants, crossover numbers stayed at wild type levels, while the number of non-crossovers was decreased. This finding suggests that in order to maintain the obligate crossover, the ratio of crossovers to non-crossovers (CO:NCO) must change. It has also been proposed that there is a decision point that occurs at an early stage, at or before the appearance of stable strand exchanges, that determines the pathway by which DSBs will be repaired (Borner et al. 2004). Crossover assurance seems to be able to influence this decision towards a crossover when DSBs are reduced in order to maintain the obligate crossover.

Figure 8. Crossover Assurance. A. If the cell were to obtain three crossovers and those crossovers were to be placed randomly among the chromosomes, it is likely that the larger one would obtain all three. B. Under the assumption of crossover assurance, those 3 crossovers would be evenly distributed among the chromosomes, such that all homologous pairs receive at least one crossover.

As described earlier, it is possible for homologs to fail to obtain the obligate crossover. Chromosome pairs lacking the obligate crossover are termed E_0 chromosomes (see Figure 8A for a pictorial example). These occur when the controls on crossover formation fail to ensure the obligate crossover, and can result in nondisjunction of the E_0 chromosome pair (see figure 5). Again, nondisjunction results in aneuploid gametes, and if the gametes are used in fertilization, they can result in aneuploid offspring.

Many proteins have been shown to be involved in the crossover assurance mechanism. For example, Ndj1 is a protein involved in tethering telomeres to the nuclear envelope, and mutations of this protein showed an increase in the incidence of E_0 's, suggesting a failure of crossover assurance (Chua and Roeder 1997). Mutations in all characterized ZMM proteins show a decrease in crossover assurance as well (Shinohara et al. 2008). ZMM (Zip, Msh, Mer) proteins are a family of meiosis specific proteins that coordinate recombination and SC formation in *S. cerevisiae*. All zmm mutants show defective SC formation along with a reduction in crossovers.

In this thesis, crossover assurance is assayed by determining the incidence of E_0 chromosomes. Heterozygous genetic markers placed along the length of a chromosome can allow detection of all crossovers between a homologous pair. This information can then be used to determine how many, if any, crossovers were formed along the length of those homologs.

Crossover interference

Crossover interference describes the observation that in the event of multiple crossovers between homologous chromosomes, those crossovers will be more widely spaced than random expectations would suggest (Figure 9). Double strand break events are the pre-cursors to crossover formation, however there are many more DSB events than there are crossovers. Only a subset of DSBs is repaired as crossovers and those crossovers seem to be evenly spaced along

the length of each chromosome. This even spacing suggests some sort of inhibition, or interference, of crossover formation in the region surrounding a crossover event. This phenomenon was first seen in *Drosophila melanogaster* (Muller 1916) and has been shown to function across large distances, in some cases an entire chromosome (Hillers and Villeneuve 2003).

Figure 9. Crossover interference. A. If the homologous pair is going to receive two crossovers and those crossovers are distributed randomly, it is possible for them to be placed very near each other on the chromosome arm. B. Observation of crossovers in many organisms shows that those two crossovers are not randomly placed, and instead are evenly spaced along the chromosome arm.

Interference suggests that a crossover event either influences, or is influenced by, another crossover event. Shinohara et al. 2003 has suggested that there are three functions that interference must perform. First, crossovers or pre-crossover intermediates trigger a signal. Second, the signal must be relayed from the triggering event to the target event. Third, something must ensure that the target event(s) form non-crossovers rather than crossovers. One model that describes how these functions are implemented suggests that the interference signal begins at a crossover site and spreads outward along the SC, blocking crossovers in adjacent intervals (Egel 1978; Maguire 1988; King and Mortimer 1990; Sym and Roeder 1994; Kaback et al. 1999). Another model suggests that axial stress is placed on meiotic bivalents to promote crossover formation. Once a crossover forms, axial stress is relieved locally, preventing nearby intermediates from becoming crossovers (Storlazzi et al. 1996; Zickler and Kleckner 1999).

Many proteins involved in crossover assurance are also involved in crossover interference. For example, *ndj1* mutants (Chua and Roeder 1997) and most of the *zmm* mutants

show defects in both crossover assurance and in crossover interference, supporting the theory that the two controls are mechanistically linked. However, mutation in a newly characterized ZMM protein, Spo16, suggests that the two mechanisms can be separated (Shinohara et al. 2008). Spo16 works with Spo22 (aka Zip4) to promote assembly of the helicases Msh4 and Msh5 on chromosomes. *spo16* mutants showed defects in synaptonemal complex formation, a 50-60% reduction in crossover formation, and wild type levels of non-crossovers. The shift in CO:NCO ratio (discussed in Crossover Assurance) suggests an inability to maintain crossover homeostasis and increases the likelihood of E_0 chromosomes. Crossover assurance is defective in the *spo16* mutant, however the residual crossovers do show interference. The *spo16* mutant is the only characterized ZMM protein that shows a functional interference mechanism in the absence of crossover assurance. Another protein, Tid1, is involved in promoting the assembly of recombinases at DSB sites (Shinohara et al. 2000). Mutations in *TIDI* have shown a reduction in crossover interference, however the effect on crossover assurance is unknown (Shinohara et al. 2003).

In this thesis, crossover interference is assayed by utilizing heterozygous genetic markers along the length of a chromosome to detect crossovers in each interval. Crossover interference can be detected by determining the extent to which one crossover event influences the likelihood of having a crossover event nearby.

Distributive disjunction

Although crossovers are the most effective way to ensure proper homolog disjunction, some organisms have a back up mechanism for segregating non-recombinant chromosomes. This phenomenon of properly segregating E_0 chromosomes has been termed distributive disjunction. One example of this can be seen in chromosome 4 in *Drosophila*. Chromosome 4 is always a

nonexchange chromosome (Sturtevant 1951), yet disjunction of the fourth chromosome occurs in approximately 99.9% of meioses (Baker and Carpenter 1972). Distributive disjunction has also been seen in yeast. Dawson et al. (1986) found that although yeast artificial chromosomes (YACs) formed crossovers at a lower level than native homologs, they showed wild type levels of disjunction. Although distributive disjunction appears to be functional in *S. cerevisiae*, it is roughly 100 times less efficient than in *Drosophila*. Two nonhomologous chromosomes in *S. cerevisiae* are segregated from each other in approximately 90% of meioses (Guacci and Kaback 1991; Loidl et al. 1994), compared to the 99.9% in *Drosophila*. The mechanism by which distributive disjunction functions in yeast is poorly understood. Pairing does seem to occur between nonhomologous chromosomes (Loidl et al. 1994), however, this pairing is an unusual structure distinct from the SC during meiosis I.

Study Organism and Proteins of interest

Saccharomyces cerevisiae

Saccharomyces cerevisiae is a convenient organism in which to study meiosis for many reasons. Yeast are eukaryotes and many meiotic processes are conserved within the eukaryotic domain. They are single celled organisms with about a 90-minute mitotic cell cycle, which makes them very easy to keep and grow in a laboratory environment. They can exist in both haploid and diploid forms and have two haploid mating types. Mating type is determined by the mating type gene on chromosome III which has two alleles: a and α . They have a sequenced genome, making site directed manipulation and genetic marker insertion possible. Probably the most important quality of yeast to meiotic studies is that there is some aneuploid cell survival. For example, a haploid yeast that has an extra copy of one of its chromosomes will be able to survive (See figure 5). However, if the haploid is missing one of its chromosomes, all of the

genetic information encoded by that chromosome is also missing, and the cell will not survive. In the context of nondisjunction, when both homologs are pulled to the same pole during meiosis I, two daughter cells are left missing a copy of that chromosome. In yeast, these cells do not survive, while the daughter cells that have two copies of that chromosome are able to survive and replicate. In this way, spore viability can be used as an indirect readout of nondisjunction in yeast. A tetrad with only two viable spores is an indication that one homologous pair was missegregated, leaving two meiotic products missing a copy of the missegregated chromosome. Likewise, a tetrad with zero viable spores is an indication that two or more homologous pairs were missegregated, potentially leaving all four meiotic products missing at least one chromosome.

Two proteins that are of interest in the Hillers lab, Ndj1 and Tid1, have been previously demonstrated to play roles in crossover control during meiosis in *Saccharomyces cerevisiae*. Both Ndj1 and Tid1 have been shown to play roles in crossover interference (Chua and Roeder 1997; Shinohara et al. 2003). Mutations in *NDJ1* have also shown an elevated incidence of E_0 and nondisjunction in meiosis I, indicating a role for Ndj1 in crossover assurance and distributive disjunction. However, mutations in *TID1* do not show the same pattern of nondisjunction as *ndj1* mutants. This difference suggests Tid1 may not play a role in crossover assurance or distributive disjunction.

Ndj1

One of the most important steps in meiosis is the search for homologs. Without homologous chromosome interaction, no crossovers can be made, and without that, efficiency of disjunction is reduced. The mechanism by which homologs find their partners has been well studied in *Saccharomyces cerevisiae*. During vegetative growth, telomeres cluster as many aggregates at the nuclear periphery. Following DNA replication, telomeres detach from the nuclear envelope and centromeres attach and cluster at the spindle pole body (SPB), the yeast equivalent of the centrosome. This cluster is called a Rabl orientation (Figure 10). During leptotene, when DSBs form, centromeres are released and telomeres become re-associated with the nuclear envelope in a few small aggregates. During the transition from leptotene to zygotene, the telomeres begin to cluster at the SPB, forming the bouquet (Figure 10). During zygotene, telomeres begin to move about the nuclear periphery (Figure 10). This movement may play a role in the search for homologs, as well as the pairing and untangling of homologs. At the same time, axial elements begin to pair and synapse at DSB sites and dHJs begin to form. During pachytene, while dHJs are resolved and recombination is completed, the chromosomes appear dispersed around the nuclear envelope (Figure 10). The SC is dismantled during diplotene, leaving the homologs only connected by mature chiasmata.

Telomere movements in prophase I of *S. cerevisiae* meiosis are partly dependent on the telomere binding protein Ndj1 (Scherthan et al. 2007). Ndj1, also known as Tam1 (Telomere associated meiotic protein), is a protein that is expressed during meiosis, localizes at telomeres and aids in the pairing of homologs in the nucleus (Chua and Roeder 1997). Ndj1 is encoded by the open reading frame *YOL104C* on chromosome XV, has a 2.4 kb mRNA, and is 352 amino acids in length with no similarity to any other known proteins (*Saccharomyces* Genome Database). Ndj1 functions with the SUN domain proteins Mps3 (Conrad et al. 2007) and Csm4

(Conrad et al. 2008). SUN domain proteins have also been implicated in normal meiotic telomere behavior in *S. pombe* (Miki et al. 2004; Shimanuki et al. 1997; Chikashige et al. 2006), mouse (Ding et al. 2007; Schmitt et al. 2007) and *C. elegans* (Penkner et al. 2007).

Figure 10. Chromosomal movement during prophase I. The Rabl orientation is where centromeres (green balls) are pulled to the spindle pole (dark blue structure with emanating microtubules, anchored in the nuclear membrane), with the telomeres (red balls) not connected. During leptotene, centromeres move away from the pole while telomeres attach to the nuclear membrane and move to a small area adjacent to the spindle pole, forming the bouquet. During zygotene, chromosomes move around the nuclear envelope as the synaptonemal complex forms (red ladder-like structure). During pachytene, synapsis is completed, dHJs are resolved and recombination is completed. (Dresser 2008).

Mutations in *NDJ1* result in delays in pairing, axial element formation, SC formation, and the overall first meiotic division (Trelles-Sticken et al. 2000). These effects center on the failure of telomeres to re-tether to the nuclear envelope to form the bouquet. The mutation does not affect the dissolution of centromere clusters or vegetative telomere clusters; however, it does hinder telomere positioning at the bouquet (Trelles-Sticken et al. 2000). Reduced movement was

seen in *ndj1* mutant cells, suggesting chromosomes must be tethered to the nuclear envelope to get maximal movement (Scherthan et al. 2007). The initiation of DSB α (resection and strand invasion – see Figure 7) was not impaired, however dHJ intermediates were delayed (Wu and Burgess 2006).

The SUN protein, Mps3, is required for spindle pole body duplication and chromatid cohesion. It interacts with Ndj1 to allow for tethering of telomeres to the nuclear envelope. Removal of the Ndj1 interaction site on Mps3 gives an *ndj1*-like separation of function allele (Conrad et al. 2007). Csm4 interacts with Ndj1 to aid telomere movement to the bouquet. In the absence of Csm4, Ndj1 bound telomeres can tether to the nuclear envelope, but cannot form the cluster of the bouquet (Kosaka et al. 2008). These results suggest a 2-step process in which Ndj1 allows for tethering to the nuclear envelope and Csm4 allows the movement of those telomeres along the nuclear envelope into the bouquet formation. Csm4 may also promote efficient second-end capture of DSBs following the homolog search, as well as resolution of dHJs to crossovers (Kosaka et al. 2008).

Mutations of *Ndj1* lead to an increase in nondisjunction, suggesting a defect in the known pathways of crossover control in addition to the back-up mechanism, distributive disjunction. Recombination does not seem to be impaired, however reduced crossover interference and crossover assurance was observed (Chua and Roeder 1997). There was no significant effect of this mutation in mitosis, however sporulation efficiency and spore viability were significantly reduced from wild type.

To test the functionality of the distributive disjunction pathway in *ndj1* mutants, Conrad et al. (1997) made strains with one homologous pair replaced with yeast artificial chromosomes (YACs). The homologs in these strains recombine and have wild type disjunction rates. The YACs do not recombine, but can segregate using distributive disjunction in wild type cells.

YACs set up a forced achiasmate (E_0) chromosome. The YACs will not be segregated by normal disjunction utilizing chiasmata, but instead will only segregate by a functional distributive disjunction mechanism. They found that in wild type cells, the YACs disjoin correctly in 89% of meioses, and in *ndj1* mutant cells, YACs were segregated in 62% of meioses (random segregation would be 50%). These results show that Ndj1p is involved in crossover interference, crossover assurance, and distributive disjunction of achiasmate chromosomes.

Tid1

During DSB repair Rad51, a homolog of the recombinase RecA (a bacterial strand exchange protein), has been shown to promote homologous strand invasion by forming right-handed helical filaments on single stranded DNA (ssDNA) at DSB sites (the 3' overhang left after resection). Rad51 has been shown cytologically to associate with Dmc1, also a RecA homolog, at hotspots for recombination (Shinohara et al. 1997). However, when no DSBs are present, Dmc1 can be found associating with double stranded DNA (dsDNA). There is less biochemical data on the function of Dmc1, however the human Dmc1 has been shown to promote the formation of homologous joint molecules to form toroidal oligomers on DNA (Masson et al. 1999 and Li et al. 1997). Deletion studies have shown that *dmc1* single mutants do not form the central element of the SC (Bascom-Slack et al. 1997). These mutants also showed defective strand invasion and crossover interference, however the overall levels of meiotic recombination were similar to the wild type strain. Overexpression of Rad51 has been shown to rescue the effects of a *dmc1* mutation; likewise, overexpression of Dmc1 has been shown to rescue a *rad51* mutation (Shinohara et al. 2000).

Recombination accessory factors may stimulate crossovers by directing recombinases to double strand break sites early in meiosis. Tid1, also known as Rdh54, is a translocase and a

recombination accessory factor in the Swi2/Snf family of helicase-related proteins (Eisen et al. 1995). Tid1 can translocate along DNA at 80 bp/s expending one ATP per bp (Nimonkar *et al.* 2007). Tid1 promotes the assembly of Dmc1 to DSB sites by exposing ssDNA through its local unwinding of dsDNA (Shinohara et al. 2003) and dissociating Dmc1 from dsDNA during meiosis (Holzen et al. 2006).

Tid1p seems to be present throughout the cell cycle, however mutations only show effects during meiosis. Mutations of Tid1 have shown a failure to release Dmc1 from dsDNA and a delay in DSB repair (Shinohara et al. 1997). A *tid1* null mutation showed further delays in the appearance of Zip1, a protein involved in the central region of the SC, and delays in the dissociation of the SC (Shinohara et al. 2003). This same study showed that the levels of crossovers and non-crossovers between homologs were similar to wild type levels, but there was a reduction in crossover interference. Double mutation of *tid1* and *rad54* has shown an accumulation of DSBs and a reduction in crossover formation (Shinohara et al. 2003). The spore viability patterns of the *tid1* mutants in each of these studies do not show the typical pattern indicative of nondisjunction (elevated levels of 2- and 0-spore viable tetrads), which suggests that crossover assurance may still be functional in the absence of Tid1.

Previous studies

Our lab's initial studies aimed to elucidate the differences in spore viability in *tid1* and *ndj1* mutants seen by two previous studies. Chua and Roeder (1997) showed an increase in 2 and 0 spore viable tetrads in an *ndj1* mutant, indicative of Meiosis I nondisjunction (see nondisjunction and aneuploidy for explanation). They also showed that a mutation in Ndj1 produced defective crossover interference as well as crossover assurance. Conrad *et al.* (1997) showed that *ndj1* mutants could not segregate achiasmate chromosomes, suggesting a role for Ndj1p in distributive disjunction. Shinohara *et al.* (2003) showed that *tid1* mutants produced a defect in crossover interference, without significantly reducing recombination. The spore viability pattern seen in this mutant does not follow the same pattern typically indicative of problems with disjunction. Instead, it shows reduced spore viability without altering the distribution of viable tetrads from that of the wild type. However, this study did not directly assay crossover assurance, or the incidence of E₀ chromosomes. We first wanted to determine if the spore viabilities seen in previous studies could be reproduced in the same strain background. Following this initial study, we set out to answer two questions: [1] Is Distributive disjunction functional in *tid1* mutants? and [2] Is crossover assurance functional in *tid1* mutants?

Assay of Spore Viability Patterns

The Hillers lab is interested in meiotic crossover control, and in particular two proteins involved in the proper disjunction of homologs, Ndj1 and Tid1. Ndj1 plays a role in crossover interference, crossover assurance and distributive disjunction. The spore viability pattern of *ndj1* shows a clear indication of meiosis I nondisjunction. Tid1 has been shown to play a role in crossover interference, however the spore viability pattern of *tid1* mutants suggest either a functional crossover assurance or distributive disjunction mechanism. We attempted to

reproduce these spore viability patterns in the SK1 strain background. Both mutants showed similar spore viability patterns to the previously published data (See Figure 11). Wild type yeast showed a high percentage of 4 spore viable tetrads and low percentage of 3, 2, 1, and 0 spore viable tetrads. The *ndj1* mutant shows a reduction in 4 spore viable, and an increase in 2 and 0 spore viable tetrads compared to wild type (Figure 11, Table 1, $2 \times 5 \chi^2 p < 0.001$). This distribution of spore viability is indicative of meiosis I nondisjunction; 2 spore viable being an indication of nondisjunction of one homologous pair, and 0 spore viable indicating nondisjunction of two homologous pairs in one meiotic event. The *tid1* mutant showed a significant difference from wild type and the *ndj1* mutant ($2 \times 5 \chi^2 p < 0.001$). Overall spore viability was decreased, with a reduction in 4 spore viable tetrads, an increase in 3, 2 and 1 spore viable tetrads, and no change in 0 spore viable tetrads compared to wild type (Figure 11). This spore viability pattern shows functional disjunction in meiosis I and suggests that either crossover assurance or distributive disjunction may be intact in *tid1* mutants.

Table 1. SK1 spore viability

Strain	Number of asci analyzed	Spore Viability				
		4 s.v.	3 s.v.	2 s.v.	1 s.v.	0 s.v.
WT	136	115	12	4	4	1
	(%)	84.6	8.8	2.9	2.9	0.7
<i>ndj1</i>	116	54	12	22	10	18
	(%)	46.6	10.3	19	8.6	15.5
<i>tid1</i>	136	55	39	27	14	1
	(%)	40.4	28.7	19.9	10.3	0.7

Figure 11. Percent spore viability of SK1 native strains. Values are shown in table 1. Error bars are 95% confidence intervals. Distribution of spore viability in both the *ndj1* and *tid1* mutants is significantly different from wild type, and the two mutants are significantly different from each other (2×10^{-2} , $p < 0.001$).

Assay of Distributive disjunction

To determine if distributive disjunction is functional in *tid1* mutants, we looked at the segregation of E_0 chromosomes. Jon Henzel performed this work for his thesis (completed May 2010). For this study, homeolog strains were constructed in each of the wild type, *ndj1* and *tid1* mutant strains. These homeologs were diploids with all *S. cerevisiae* chromosomes, with the following exceptions: one chromosome V in strain S288C was replaced with chromosome V from *S. paradoxus*, and one chromosome III in strain Y55 was replaced with a chromosome III from *S. paradoxus*. *S. cerevisiae* and *S. paradoxus* chromosomes V are syntenic, but only show 75-80% sequence identity. These homeologs fail to cross over in roughly 99% of meioses. This inability to crossover sets up a forced achiasmate chromosome pair, similar to that of the YACs used by Conrad et al. (1997), as discussed earlier. This allows us to assay the ability of these mutants to segregate achiasmate chromosomes through spore viability. If the mutant is able to segregate achiasmate chromosomes, we expect to see a spore viability pattern similar to wild type.

If they cannot segregate achiasmate chromosomes, we expect to see a reduction of 4 spore viable tetrads and an increase in the 2 and 0 spore viable tetrads indicative of meiosis I nondisjunction.

In the Y55 homeolog, the two mutants had spore viability patterns that are each significantly different than wild type (Figure 12, Table 2). The *ndj1* mutant showed elevated 2 and 0 spore viable tetrads relative to 3 and 1 spore viable tetrads. In the *tid1* mutant, 3 and 1 spore viable levels were both higher than 2 spore viable tetrads. These patterns suggest that *ndj1* mutants show an inability to segregate the homeologous chromosomes. The *tid1* mutant showed a reduction in spore viability, but shows less of a defect in segregating homeologous chromosomes.

In the S288C homeologs, the *tid1* mutant showed a wild type distribution of spore viability (Fig 13, Table 3), while the *ndj1* mutant showed an increase in 2 and 0 spore viable tetrads, indicative of a defect in distributive disjunction. The overall spore viability of the *ndj1* homeolog was different from wild type in both strain backgrounds, and different from the *tid1* homeolog in S288C (2×5^{-2} , $p \ll 0.001$). The differences seen in overall spore viability may reflect underlying genetic differences between Y55 and S288C.

Table 2. Y55 homeolog spore viability

Strain	Number of asci analyzed	Spore Viability				
		4 s.v.	3 s.v.	2 s.v.	1 s.v.	0 s.v.
WT	214	154	35	16	3	6
	(%)	72	16.3	7.5	1.4	2.8
<i>ndj1</i>	219	75	32	44	26	42
	(%)	34.2	14.6	20.1	11.9	19.2
<i>tid1</i>	122	33	29	15	18	27
	(%)	27.0	23.8	12.3	14.8	22.1

Figure 12. Percent spore viability of Y55 homeolog strains. Values are shown in table 2. Error bars are 95% confidence intervals. Overall distribution of *ndj1* and *tid1* mutants are both significantly different from wild type (2×10^{-2} , $p < 0.001$); the two mutants are not significantly different from each other.

Table 3. S288C homeolog spore viability

Strain	Number of asci analyzed	Spore Viability				
		4 s.v.	3 s.v.	2 s.v.	1 s.v.	0 s.v.
WT	123	73	19	7	4	20
	(%)	59.4	15.4	5.7	3.2	16.3
<i>ndj1</i>	128	6	9	29	13	71
	(%)	4.7	7.0	22.7	10.1	55.5
<i>tid1</i>	132	57	37	8	3	27
	(%)	43.2	28.0	6.1	2.3	20.4

Figure 13. Percent spore viability of S288C homeolog strains. Values are shown in Table 3. Error bars are 95% confidence intervals. The *ndj1* mutant is significantly different from both WT and the *tid1* mutant ($p < 0.001$); the *tid1* mutant is not significantly different from WT.

Ndj1 plays a role in crossover interference, crossover assurance and distributive disjunction (Chua and Roeder 1997; Conrad et al. 1997). The spore viability pattern of the *ndj1* mutant shows a clear indication of meiosis I nondisjunction. Tid1 has been shown to play a role in crossover interference (Shinohara et al 2003), however the spore viability pattern of the *tid1* mutant suggest either a functional crossover assurance or distributive disjunction mechanism. We were able to reproduce these findings in *ndj1* and *tid1* mutants in the SK1 background. We also showed that *tid1* was better able to segregate achiasmate chromosomes in homeolog strains than the *ndj1* mutant, suggesting a functional distributive disjunction mechanism in *tid1* mutants. This study, however, did not assay crossover assurance. To determine if crossover assurance is functional in a *tid1* mutant, heterozygous markers along the length of a chromosome in native strains of WT, *ndj1* and *tid1* mutants were used to determine the incidence of E_0 . This is a direct assay of crossover assurance because the presence of E_0 's is a result of a failure in the crossover control mechanisms. We find that the incidence of E_0 chromosomes is increased in both the *ndj1* and *tid1* mutant strains. These findings show that the differences seen in the spore viability

patterns of *ndj1* and *tid1* mutants are not due to differences in crossover assurance, but due to differential effects on distributive disjunction. We suggest that Ndj1, but not Tid1, plays a role in distributive disjunction, the cells' back-up segregation method.

Materials and Methods

Yeast Strains

Both *ndj1* and *tid1* mutants were generated in the Y55 strain background to create the test strains. Non-mutant strains were used as control stains. The non-mutant test strains were derived from KHY 113 (a gift from Dr. Eva Hoffman 4/15/07) and KHY 144 (received from Rhona Borts 7/3/09). KHY 148 and 149 were used to determine mating type of haploid spores following dissection. Table 4 summarizes the genotypes of strains used in this study.

Table 4. Genotype of strains used in this study

Strain	Genotype
KHY 310	<i>ura3-</i> , <i>his4-</i> , <i>LEU2</i> , <i>lys2-</i> , <i>ycl056c::URA3</i> , <i>ycl047c::KAN^R</i> , <i>ycr076c::LYS2</i> , <i>MAT</i>
KHY 302	<i>ura3-</i> , <i>HIS4</i> , <i>leu2-</i> , <i>lys2-</i> , <i>ycr101c::HYG^R</i> , <i>MATa</i>
KHY 300	<i>ura3-</i> , <i>his4-</i> , <i>LEU2-</i> , <i>lys2-</i> , <i>ycr101c::HYG^R</i> , <i>ndj1::cloNAT</i> , <i>MAT</i>
KHY 315	<i>ura3-</i> , <i>HIS4-</i> , <i>leu2-</i> , <i>lys2-</i> , <i>ycl056c::URA3</i> , <i>ycl047c::KAN^R</i> , <i>ycr076c::LYS2</i> , <i>ndj1::cloNAT</i> , <i>MATa</i>
KHY 321	<i>ura3-</i> , <i>his4-</i> , <i>LEU2-</i> , <i>lys2-</i> , <i>ycr101c::HYG^R</i> , <i>tid1::cloNAT</i> , <i>MAT</i>
KHY 319	<i>ura3-</i> , <i>HIS4-</i> , <i>leu2-</i> , <i>lys2-</i> , <i>ycl056c::URA3</i> , <i>ycl047c::KAN^R</i> , <i>ycr076c::LYS2</i> , <i>tid1::cloNAT</i> , <i>MATa</i>

Media

Yeast strains were maintained in Petri dishes containing rich medium YPAD (1% Yeast Extract, 2% peptone (tryptone), 2% dextrose, 1.75% agar and 0.004% adenine sulfate). Selection of antibiotic resistance occurred on YPAD plus either 100 μ L or 200 μ L of the appropriate antibiotic (final concentration of 0.5mg/mL (2.5mg per plate) of Geneticin to test for the Kan resistance marker, 0.125 mg/mL (10 mg per plate) of nourseothricin for the *cloNAT* resistance marker, and 0.3 mg/mL (6 mg per plate) of Hygromycin B to test for the Hygromycin resistance marker). Synthetic dropout media (SD) was used to select for auxotrophic strains (0.64% Difco YNB, 2% dextrose, 1.75% agar plus appropriate amino acids. Nitrogen-deficient plates (SPO) were used to induce sporulation of diploid yeast strains (1% potassium acetate,

0.1% yeast extract, 0.05% dextrose, 0.025% complete amino acid mix). LB broth was used for bacterial growth for plasmid minipreps (1% tryptone, 0.5% yeast extract, 0.5% NaCl). TE buffer was used to store extracted DNA (10 mM Tris pH 8.0, 1 mM EDTA).

Gene Knockouts

A cassette conferring nourseothricin (cloNAT) antibiotic resistance was inserted into each gene of interest in this study. Nourseothricin is a polyketide antifungal compound produced naturally by the bacterium *Streptomyces noursei*. The cloNAT resistance cassette was cloned into the open reading frame of *TID1* using pBluescriptII *tid1::CloNAT* plasmid built by Elaine Morlock. The CloNAT cassette from this plasmid was cut out using restriction endonucleases and transformed into the *TID1* open reading frame by transformation. A 1 kb region of the *TID1* open reading frame was replaced with the CloNAT resistance gene in the antiparallel orientation. The open reading frame of *NDJ1* was completely replaced with the CloNAT cassette from pAG25. Figure 14 diagrams the PCR mediated gene insertion used for the *ndj1::CloNAT* disruption. This same method was used for *ycl056c::URA3*, *ycl047c::KAN^R* and for *ycr101c::HYG^R* marker insertions (see Table 5 for plasmids in these insertions). Transformed yeast cells were selected for on antibiotic plates and confirmed with PCR.

Figure 14. Diagram of gene knockout. The genetic marker to be inserted was amplified with insertion site-specific markers. This PCR product was then used as the transforming DNA and site-specifically inserted into the target gene. The genetic marker usually replaced most or all of the gene to be mutated, rendering the original gene site non-functional.

Genetic Markers

Both antibiotic resistance genes and nutritional genes were used as genetic markers at various loci along the length of chromosome III.

Figure 15. Genetic markers used in the crossover assurance assay.

Antibiotics. A cassette conferring Geneticin (G418) resistance was inserted into *YCL047C*.

Geneticin is an aminoglycoside antibiotic and is naturally produced by the gram-positive bacterium *Micromonospora rhodorangea*. Geneticin inhibits peptide synthesis by inhibiting the elongation step in both prokaryotes and eukaryotes. A cassette conferring Hygromycin (Hygromycin B) resistance was inserted into *YCR101C*. Hygromycin is an aminoglycoside antibiotic produced by the bacterium *Streptomyces hygrosopicus* that kills bacteria, fungi and

higher eukaryotic cells by inhibiting protein synthesis. A cassette conferring Nourseothricin (CloNAT) resistance was inserted into both *TID1* and *NDJ1* genes in separate strains.

Nourseothricin is a streptothricin-class antibiotic that kills bacteria, mycobacteria, mycoplasmas, protozoae, yeasts, viruses, and plants through inhibition of ribosomal protein synthesis and induction of miscoding.

Nutritional. A cassette conferring the *URA3* gene was inserted into *YCL056C*. The *LYS2* gene was amplified from KHY 148 and inserted into *YCR076C*. *HIS4* and *LEU2* were at their natural genetic loci.

Transformations

Yeast gene transformations were performed via one-step gene replacement as described below as well as in Hillers Lab Procedures manual. All antibiotic resistance cassettes required a 12-24 hour outgrowth at 30°C in rich YPAD media prior to plating onto YPAD+antibiotic for selection.

Transforming DNA. Inserts for use in transformations were amplified genes from known plasmids, except for *ycr076c::LYS2*. The genes were amplified with primers containing tails specific to the insertion site in *S. cerevisiae*. PCR reactions were 50 µL reactions and included 10 µL 5x GoTaq Buffer, 1 µL dNTPs, 5 µL MgCl₂, 1 µL of each 5µM primer, 0.25 µL template (plasmid), 1 µL TaqPol, and 30.75 µL water. All PCR reactions were run with an initial denature step for 10 min at 94 °C, followed by 35 cycles of another denature step at 94 °C for 2 min, an annealing step for 30 seconds at the primer's calculated melting point, and an extension step at 72 °C for an amount of time based on amplicon length. After the PCR cycling, the reactions were

held at 4 °C, and stored at -20 °C. Table 5 shows the source of each amplicon, as well as the primers used and their specific PCR conditions.

Table 5. Transforming DNA

Transforming DNA	Source of transforming DNA	Primers	Annealing Temp	Extension Time (min)	Size
<i>ycr056c::URA3</i>	pAG60	YCL056c F and R	52C	2:30	1.6 kb
<i>ycl047c::KAN^R</i>	pUG6	YCL047C 3' and 5'	60C	2:00	1763 bp
<i>ycr076c::LYS2</i>	KHY 148	*			
<i>ycr101c::HYG^R</i>	pAG 32	YCR101c F and R	61C	2:00	1.8 kb
<i>tid1::cloNAT</i>	pBluescript II tid1::cloNAT	**			
<i>ndj1::cloNAT</i>	pAG25	Ndj1Kan64 F and R	66C	1:30	1432 bp

*See *ycl076c::LYS2* stitch section and Table 6.

**digested with NotI (not a PCR product)

Glycerol Yeast Transformation. Yeast cells were grown in YPD liquid media with adenine supplement (YPAD) overnight at 30 °C. The next day, 25 mL of YPAD was inoculated with 1 mL of the overnight culture, and allowed to grow at 30 °C until the cultures reached an optical density at 600 nm between 0.4 - 0.6 A. The cells were then pelleted in 50 mL conical tubes for 3 minutes at 3000 RPM and the supernatant was decanted. The pellet was then washed in 25 mL water and 10 mL Li-T (100mM lithium acetate, 10 mM Tris pH 7.5). The cells were pelleted for 3 minutes at 3000 RPM and the supernatant was removed between each wash. The final pellet was then re-suspended in 1 mL 100 mM lithium acetate (LiOAc) and transferred to a microcentrifuge tube. This was then pelleted for 30 seconds at max speed and supernatant was removed. Pellet was resuspended in 100 mM lithium acetate such that each transformation would get 50 µL of culture. 50 µL of each culture was then aliquoted to fresh microcentrifuge tubes, and pelleted to remove the supernatant. Each pellet was then re-suspended in 325 µL master-mix (per transformation: 240 µL 50% PEG 3350, 36 µL 1M LiOAc, and 50 µL salmon sperm (boiled at 95 °C for 5 min)). 30 µL of the PCR product was then added and heat shocked for 5 min at 42°C,

then incubated at room temperature for 15 min with slow inverting. 8 μ L 60% glycerol was then added and the solution was heat shocked at 42 °C for 15 min. The cultures were pelleted 2 min at 3000 RPM and resuspended in water for plating. Transformations with a nutritional marker were plated immediately, while antibiotic resistance markers were added to 3 mL YPAD for an overnight outgrowth. Table 6 shows the PCR product markers, their specific transformation parameters, and media for selection of transformants. For all transformations, full plasmids were added to positive controls in place of PCR products, and negative controls contained no transforming DNA. All other conditions of the positive and negative controls were the same as the transformant tubes.

Transformation by Electroporation. Yeast cells were grown in YPD liquid media with adenine supplement (YPAD) overnight at 30 °C with shaking at 225 rpm. The next day, 25 mL of YPAD was inoculated with 750 mL of the overnight culture, and allowed to grow at 30 °C until the cultures reached an optical density at 600 nm between 0.4 - 0.6 A. The cells were then pelleted in 50 mL conical tubes for 3 minutes at 1500 RPM and the supernatant was decanted. The cells were then re-suspended in 1.8 mL TE pH 7.5 and transferred to a 15 mL conical. 200 μ L 1M LiOAc was added and the mixture was Incubated 45 minutes at 30C with shaking at 50 rpm. 100 μ L 0.5M DTT was added and continued shaking at 50 rpm for 15 minutes. 8mL ddH₂O was added to the culture, spun 1500 rpm for 3 minutes and the supernatant decanted. The pellet was re-suspended in 10mL ddH₂O, spun at 1500 rpm for 3 minutes, and the supernatant was decanted. The pellet was then re-suspended in 500 μ L cold 1M sorbitol and transferred to a 1.5 mL eppendorf microcentrifuge tube, spun 15 seconds at max speed (13.2 krpm) and the supernatant aspirated. The final pellet was re-suspended in 1 M cold sorbitol such that each transformant got 40 μ L. Electroporation cuvettes were cleaned with 70% EtOH prior to the

procedure, and kept on ice for the duration of the procedure. 40 µL of the cell slurry, 2.5 µL 10mg/mL boiled salmon sperm DNA and various concentrations of the transforming DNA (5-12 µL) were added to the electroporation cuvettes (See Table 2 for transforming DNA for controls and experimental transformations; no DNA was added for the negative control). Tubes were tapped on the table (to get contents to bottom), wiped with a Kim wipe to remove condensation, and electroporated at 1.5 kV, 25 µF, 200 (set at 129 R5). 1mL of cold 1M Sorbitol was added immediately. Samples were plated to selective media plates prepped with 100 µL 1M sorbitol, according to the DNA used.

An example of plating methods when transforming DNA was *ycr076c::LYS2* stitch and the positive control was pAG26 follows. All samples were transferred to 1.5 mL eppendorf tubes and spun at 8krpm for 0.5 minute and the supernatant was decanted. The negative control pellet was re-suspended in 150 µL 1 M cold sorbitol and plated to one SD-lys plate (prepped with 100 µL cold sorbitol; plated with glass beads). The transformant pellet was resuspended in 300 µL cold 1M sorbitol and plated to 2 prepped SD-lys plates (150 µL for each plate). The positive control pellet was re-suspended in 150 µL 1 M sorbitol, transferred to 3 mL YPAD in a test tube, and incubated overnight at 30C with shaking at 225 rpm. The next day, 500 µL of the outgrowth was spun at 4000 rpm for 2 minutes. The supernatant was decanted and the pellet re-suspended in 100 µL ddH₂O. All of the culture was plated to YPAD+HYG plate. All plates were incubated at 30C until colonies formed, or 1 week had passed.

Table 6. Transformation parameters

Expected transformant	Plating method	Selection Plate
<i>Tid1::cloNAT</i>	Outgrowth	YPAD + cloNAT
<i>Ndj1::cloNAT</i>	Outgrowth	YPAD + cloNAT
<i>YCL056C:: URA3</i>	Direct	SD-ura
<i>YCL047C::KAN^R</i>	Outgrowth	YPAD + G418

<i>YCR101C::HYG^R</i>	Outgrowth	YPAD + Hyg
<i>YCR076c::LYS2</i>	Direct	SD-lys
Positive control for URA3 (pAG60)	Direct	SD-ura
Positive control for HYG ^R (pAG32)	Outgrowth	YPAD + Hyg

Confirmation of strains

Marker testing by selective media. All possible transformants were streaked for single colonies on the original transformation selective media (see Table 6) and incubated overnight at 30° C. A single colony from that plate was then used to start an overnight culture for gDNA prep and as the colony for colony PCR. 10 µL of each overnight culture was then plated to YPAD and grown overnight at 30C. This plate was then replicaprinted to selective media corresponding to all markers (SD-ura, SD-his, SD-leu, SD-lys, YPAD+HYG, YPAD+CloNAT, YPAD+G418, and mating type testing SD+70 and SD+227). Plates were grown overnight at 30C and checked the next day for growth/no growth.

Colony PCR. Colony PCR was used to test the transformant colonies for the correct insert. All possible transformants were streaked for single colonies on the original transformation selective media (see Table 6) and incubated overnight at 30C. The freshly grown single colonies were suspended in 25 µL 0.2 M NaOH for at least 5 min to disrupt the cell membranes, and release the DNA. PCR reactions were 15 µL reactions and included 3 µL 5x GoTaq Buffer, 0.18 µL Dntps, 0.9 µL MgCl₂, 1 µL of each primer, 1 µL template, 0.45 µL TaqPol, and 7.7 µL water. PCR reactions were then run per the primer conditions using the suspended colonies as a template. Table 7 shows the primers used for each transformation, as well as the PCR conditions for each conformation.

gDNA extraction. In some cases, colony PCR did not work and a gDNA prep extraction was necessary to isolate enough DNA to perform PCR on. The sorbitol yeast gDNA prep was used preferentially, however the glass beads prep was used as well. Both can be found in the Hillers Procedure binder and below.

Yeast sorbitol genomic DNA extraction. Yeast cells were grown overnight in liquid YPAD at 30C with shaking at 225 rpm. The next day, 1.5 mL of cultures were transferred to 1.5 mL eppendorf tubes and centrifuged at 3000 rpm for 2 minutes. The supernatant was decanted; another 1.5 mL of culture was added to the tube and re-spun. The supernatant was decanted and the pellet re-suspended in 1 mL ddH₂O and re-spun. The supernatant was aspirated off, and the pellet re-suspended in 0.5 mL 1 M sorbitol. 15 µL 0.5 M DTT and 7.5 µL 10 mM zymolyase (5 and 10 µL work just as well) was added to the mixture and the tubes were incubated at 37C in the shaking heat block (in Black lab) at 400rpm for at least 1 hour. 200 µL TE and 70 µL 10% SDS were added and the mixtures were incubated at 65C for 10 min. 320 µL 5M Potassium acetate (KoAc) was added, the tubes inverted six times and incubated on ice for 30 min. The cells were then centrifuged 6 minutes at max speed (13.2 krpm) to separate cell particles (lipids, etc) from the aqueous layer containing the DNA. While they were spinning, 2 mL eppendorf tubes were prepped with 1 mL isopropanol and 200 µL 5 M Ammonium acetate (NH₄OAc). 750 µL of the aqueous layer was transferred to fresh 1.5 mL eppendorf tubes (not the prepped 2 mL tubes), making sure to not take up any of the white pellet. The fresh tubes were spun for 5 minutes at max speed. 650 µL of the supernatant was transferred to the prepped 2 mL tubes, inverted 6 times, and centrifuged 4000 rpm for 1 minute. The supernatant was aspirated off, and the pellet dried in the vacuum centrifuge (in the UBL) for about 5 minutes, or until the pellet was dry (make sure to leave lids to tubes open). The pellet was re-suspended in 300 µL TE, 1 µL

RNase was added, and the mixture was incubated at 37C for 30 minutes. gDNA preps were stored at -20C until use.

Glass bead/Phenol yeast genomic DNA extraction. Yeast cells were grown overnight in liquid YPAD at 30C with shaking at 225 rpm. The next day, 1.5 mL of cultures were transferred to 1.5 mL eppendorf tubes and centrifuged at 3000 rpm for 2 minutes. The supernatant was decanted; another 1.5 mL of culture was added to the tube and re-spun. The supernatant was decanted and added 0.3 g glass beads, 200 μ L phenol:chloroform (gloves must be worn with phenol:chloroform), and 200 μ L Solution X-2-17 (848 μ L dd H₂O, 2 μ L 0.5 M EDTA pH 8.0, 1.0M Tris pH 8.0, 10 μ L 5 M NaCl, 100 μ L 10 % SDS, and 20 μ L Triton X-100). The mixture was vortexed 3 minutes at highest setting. 200 μ L TE was added, and the mixture was centrifuged 3 minutes at max speed. approximately 400-500 μ L of the top aqueous layer was transferred to fresh 1.5 mL eppendorf tubes. Phenol:chloroform extractions were performed 2 more times (addition of Phenol:Chloroform, vortex, centrifuge, and transfer of top aqueous layer). The DNA was then ethanol precipitated: added 0.1 volume 3M NaOAc pH5 and 2 volumes 95% EtOH and incubated 10 minutes at room temperature. The mixture was spun at max speed for 10 minutes, the supernatant decanted and pellet washed with 70% EtOH. The pellet was dried in the vacuum centrifuge, re-suspended in 50 μ L TE, and stored at -20C.

PCR test for correct inserts. PCR reactions were 15 μ L reactions and included 3 μ L 5x GoTaq Buffer, 0.18 μ L dNTPs, 0.9 μ L MgCl₂, 1 μ L of each primer, 1 μ L template, 0.3 μ L TaqPol, and 7.7 μ L ddH₂O. All PCR reactions were run with an initial denature step for 10 min at 94 °C, followed by 35 cycles of another denature step at 94 °C for 2 min, an annealing step for 30 seconds at the primer's calculated melting point, and an extension step at 72 °C for an amount of

time based on amplicon length (Table 7). After the PCR cycling, the samples were held at 4 °C, and stored at -20 °C.

Gel electrophoresis to visualize the PCRs. Gel electrophoresis was used to visualize the presence and size of amplicon from all PCR reactions. If the colony PCR did not work, sorbitol genomic DNA extractions were performed as in the Hillers Procedure notebook and that genomic DNA was used as a template in PCR. Expected sizes for both wild type and transformed DNA are shown in Table 7. Figure 16 and Table 8 shows the results of all marker tests for all strains.

Table 7. Primers and conditions for testing transformant insert.

Gene	Primers	Annealing temperature	Extension time	Expected sizes	
				WT	Trans
<i>ycl056c::URA3</i>	YCL056c Test F YCL056c Test R	58 °C	2:15 min	831 bp	2 kb
<i>ycl047c::KAN^R</i>	YCL047c Test F YCL047c Test R	55 °C	2:15 min	1348 bp	2150 bp
<i>ycl076c::LYS2</i>	YCL076c Test F2 YCL076c Test R	55 °C	1 min	No band	1042 bp
	YCL076c Test F YCL076c Test R	55 °C	5:15 min	1247 bp	5301 bp
<i>ycl101c::HYG^R</i>	YCL101c Test F YCL101c Test R	48 °C	2:15 min	482 bp	2149 bp
<i>tid1::cloNAT</i>	Tid1 F1 Tid1 R2	56.4 °C	2:15 min	2065 bp	2319 bp
<i>ndj1::cloNAT</i>	Ndj1 Test F Ndj1 Test R	53 °C	2 min	1412 bp	1656 bp

Figure 17.
Promega 1 kb
DNA ladder
(promega.com).

Figure 16. Gel visualizing and confirming all markers in all strains. 1% Agarose gel (1.5g Agarose + 150 mL 0.5xTBE). Loaded 4 μ L of all samples, and 1.5 μ L Promega 1kb DNA ladder. See Figure 17 for ladder. See Table 8 for a Gel Key.

Table 8. Gel Key.

TOP						Bottom					
Lane	Marker	Strain	Band Size (bp)	Genotype	Pg of Original PCR	Lane	Marker	Strain	Band Size (bp)	Genotype	Pg of Original PCR
1		1 kb ladder				1		1 kb ladder			
2	<i>YCL056C</i>	KHY 310	2000	<i>0563::URA3</i>	RK#4, pg 120	2	<i>TID1</i>	KHY 310	2100	WT	RK#4, pg 120
3		KHY 302	800	WT	RK#4, pg 120	3		KHY 302	2100	WT	RK#4, pg 120
4		KHY 300	800	WT	RK#4, pg 120	4		KHY 300	2100	WT	RK#4, pg 120
5		KHY 315	2000	<i>0563::URA3</i>	RK#4, pg 120	5		KHY 315	2100	WT	RK#4, pg 120
6		KHY 321	800	WT	RK #5, pg. 22	6		KHY 321	2300	<i>tid1::NAT</i>	RK #5, pg. 22
7		KHY 319	2000	<i>0563::URA3</i>	RK #5, pg. 22	7		KHY 319	2300	<i>tid1::NAT</i>	RK #5, pg. 22
8		1 kb ladder				8		1 kb ladder			
9	<i>YCL047C</i>	KHY 310	2100	<i>O47C::KAN</i>	RK#4, pg 120	9	<i>YCR076C</i> - 6	KHY 310	1000	<i>076C::LYS2</i>	RK#4, pg 120
10		KHY 302	1300	WT	RK#4, pg 120	10		KHY 302	no band	WT	RK #5, pg. 22
11		KHY 300	1300	WT	RK#4, pg 120	11		none			
12		KHY 315	2100	<i>O47C::KAN</i>	RK#4, pg 120	12		KHY 315	1000	<i>076C::LYS2</i>	RK#4, pg 120
13		KHY 321	1300	WT	RK #5, pg. 22	13		KHY 321	no band	WT	RK #5, pg. 22
14		KHY 319	2100	<i>O47C::KAN</i>	RK #5, pg. 22	14		KHY 319	1000	<i>076C::LYS2</i>	RK #5, pg. 22
15		1 kb ladder				15		1 kb ladder			
16	<i>YCR101C</i>	KHY 310	500	WT	RK#4, pg 120	16	<i>YCR076C</i> - 7	KHY 310	5000	<i>076C::LYS2</i>	RK #5, pg. 22
17		KHY 302	2000	<i>101C::HYG^R</i>	RK#4, pg 120	17		KHY 302	1200	WT	RK#4, pg 120
18		KHY 300	2000	<i>101C::HYG^R</i>	RK#4, pg 120	18		KHY 300	1200	WT	RK#4, pg 120
19		KHY 315	500	WT	RK#4, pg 120	19		none			
20		KHY 321	2000	<i>101C::HYG^R</i>	RK #5, pg. 22	20		KHY 321	1200	WT	RK #5, pg. 22
21		KHY 319	500	WT	RK #5, pg. 22	21		KHY 319	5000	<i>076C::LYS2</i>	RK #5, pg. 22
22		1 kb ladder				22		1 kb ladder			
23	<i>NDJ1</i>	KHY 310	1400	WT	RK#4, pg 120						
24		KHY 302	1400	WT	RK#4, pg 120						
25		KHY 300	1600	<i>ndj1::CloNAT</i>	RK #5, pg. 5						
26		KHY 315	1600	<i>ndj1::CloNAT</i>	RK #5, pg. 5						
27		KHY 321	1400	WT	RK #5, pg. 22						
28		KHY 319	1400	WT	RK #5, pg. 22						
29		1 kb ladder									

ycr076c::LYS2. Due to the number of markers used in this project, we ran out of pre-made markers in the pAG plasmid series. The strain background that we used had a *lys2* mutation. Because of this mutation, we were able to amplify the *LYS2* gene from another Y55 strain in the lab and use that as the insert. Using primers with 40 nt tails corresponding to *YCR076C* did not produce enough homology with the directed site, and seemed to either insert at random, or at the native *lys2* position in the genome. To add more homology to either end of the transforming DNA a PCR stitch was used to obtain the transforming DNA.

First, primers were made to amplify the *LYS2* gene from KHY 148 – *YCR076C*-5 and *YCR076C*-6 (See table 9 and Figure 18). Primers were also made to amplify regions on the right and left end of the target insertion site at *YCR076C* on chromosome III (See table 9 and Figure 18). The three pieces of DNA overlap such that the *LYS2* gene has regions of homology with regions in the *YCR076C* gene on either end. The left end of the “middle” piece (made with *YCR076C*-5 and *YCR076C*-6) is homologous with the right end of the “left” piece (made with *YCR076C*-1 and *YCR076C*-2). The right end of the “middle” piece (made with *YCR076C*-5 and *YCR076C*-6) is homologous with the left end of the “right” piece (made with *YCR076C*-3 and *YCR076C*-4). In essence, all three pieces should overlap with about 20 bp of homology between them. Once these regions were amplified using PCR the bands were visualized on an agarose gel, cut out and the DNA was purified using the QIAquick Gel extraction kit. All three PCR products were used as templates for a final PCR to stitch them together to make a final, longer PCR product. The most external primers, *YCR076C*-1 and *YCR076C*-4 (See table 9 and Figure 18), were used with the three templates.

Figure 18. Diagram of the production of the *LYS2* stitch. Specific primers amplified the *LYS2* gene and regions in the *YCR076C* region producing three pieces with overlapping regions. These three pieces were then used as templates for a final PCR stitch.

Table 9. Primers used in the *ycr076c::LYS2* stitch. Annealing temperature for all primers was 55C. Extension time for each piece was 1min/kb for Taq PCRs, and 40sec/kb for Phusion PCR's

Primer	Sequence
YCR076C-1	5'-ctgattttccctccaaggga-3'
YCR076C-2	5'-tcaccatcgcgatacaccaa-3'
YCR076C-3	5'-ttagtTTTTtagatagtatac-3'
YCR076C-4	5'-gacaccattttgcttcgcgt-3'
YCR076C-5	5'-ttggtgtatcgcgatggtgaTTGTTTCGATTTACAGCGGACG-3'
YCR076C-6	5'-CTGGCGTTAGTCTGCTAATGagacatagctatgtcgaaag-3'

*Upper case corresponds to binding in *LYS2* gene; lowercase corresponds to binding in *YCR076C*

PCR for stitch. Taq polymerase could not be used to make the three pieces of the stitch because of the addition of A's (adenine) at the 3' end of the product. This would add nucleotides to the ends of each product, and potentially interfere with the ability of the three pieces to bind during the stitch. To remedy this, Phusion® High-Fidelity DNA Polymerase was used. Phusion is a *Pyrococcus*-like enzyme fused to a processivity enhancing domain that was cloned into *E. coli* for protein purification. It has an error rate 50-fold lower than *Taq* DNA polymerase and 6-fold lower than *Pyrococcus furiosus* DNA polymerase (product website - neb.com). Phusion DNA

polymerase also has a 5' to 3' polymerase activity and a 3' to 5' exonuclease activity that will generate blunt-end products.

Phusion PCR reactions were 60 µL reactions and included 12 µL 5x Phusion HF Buffer, 1.2 µL dNTPs, 6 µL of 10 µM primer, 3 µL template, 0.6 µL Phusion DNA polymerase, and 31.2 µL ddH₂O. The 5x Phusion HF Buffer contains MgCl₂ (1.51.5mM at the final 1x reaction concentration). All PCR reactions were run with an initial denature step for 30 sec at 98 °C, followed by 30 cycles of another denature step at 98 °C for 5 sec, an annealing step for 15 seconds at 58 °C and an extension step at 72 °C for the amplicon's required time (40 sec/kb). There was a final extension step at 72 °C for 10 min. After the PCR cycling, the samples were held at 4 °C, and stored at -20 °C. Extension time was 20 sec for the right and left pieces, and 2:15 min for the middle *LYS2* piece (Figure 17).

After these pieces were made, the entirety of the samples were run on 1-1.5% 1x TAE Agarose gels, in 1x TAE, at 100 V for 1 hour. The gels were then stained for 10 min in EtBr and destained for 10 min in water. The bands were cut out of the gel using a razor blade, using a long wave UV light to visualize the bands. The bands were purified using the QIAquick Gel extraction kit. *Taq* DNA polymerase was used for the final stitch.

Tetrad dissections

Tetrad dissections were used to construct strains and also for data collection.

Selecting for Diploids. Haploid strains were first mated on YPAD by patching both haploids separately, and mixing the two haploids together (Figure 19a) and incubating overnight at 30C. This plate was then replica printed to the diploid selective media SD-his-leu; one haploid of each mating type has the *HIS4* marker, while the other has the *LEU2* marker so neither haploid can

grow on media lacking both histidine and leucine, but the diploid of the two strains can. This SD-his-leu plate was then incubated overnight at 30C. The diploid was streaked on a SD-his-leu plate, to obtain single colonies of the diploid strain. This plate was then used to pick colonies for dissection.

Sporulating. Single colonies were picked from the SD-his-leu selective plate, patched to YPAD and incubated overnight at 30C. This plate was then replica printed to SPO and incubated at 30C until the culture sporulated. A compound microscope at 400x was used to confirm the presence of tetrads. To determine the optimal sporulation time for the strains, a minimum of 2 plates were dissected over a range of days. Spore viability for each of the days was compared and the day with the highest spore viability was determined to be the optimal sporulation time. For both the WT and *ndj1* strains, the cells were allowed to sporulate for 3 days before dissection. *tid1* was sporulated for 2 days prior to dissection.

Dissection. Once the cells were ready for dissection, 5-10 μ L of cells was suspended in 10 μ L 5mg/mL zymolyase and incubated at room temperature for 8 minutes. The cells were then streaked to a YPAD plate as shown in figure 19b. The Singer MSM series 400 dissecting microscope was used to dissect the tetrads and the singer setup was used. Figure 19c shows the grid the microscope sets up for the user. The microscope actually sets up a grid of 10 columns and 9 rows, but only 9 columns were used because of how wide the streak was. I generally skipped the middle row in order to separate the tetrads to make scoring easier. The dissected plates were incubated 2 days at 30C, or until the colonies were big enough to replica print.

Figure 19. Diagram of Sporulation and Dissection. a) Example of a mating plate. b) Streak of sporulated and zymolyase digested tetrads. c) Example of a fully dissected plate.

Scoring. Dissected plates were replica printed to selective media and incubated at 30C overnight. The growth pattern of the spores was then recorded on a score sheet. For example, if a spore grew on an SD-ura plate a “+” was put in the appropriate box (Table 10). Spore viability was written in the numbered box. The spaces between markers were used to denote type of marker distribution. Parental Ditype (PD) describes marker segregation that results in only parental genotypes, or no crossover. Tetra Type (TT) describes marker segregation that results in two products with parental genotypes and two products with non-parental genotypes, or one crossover in that interval. Non-Parental Ditype (NPD) describes marker segregation that results in only products with non-parental genotypes, or a double crossover in that interval. These designations were put into an Excel spreadsheet for further analysis.

Table 10. Score sheet used to score tetrads. Examples of scoring are shown for wild type.

Strain _____ Plate # _____ Date _____

Tetrad	Spore	SD	YPAD		SD										YPAD	
		-ura		+KAN		-his		-leu		+70α		+227a		-lys		+HYG
1 4	a	+	PD	+	TT	+	PD	-	PD	+	a	-	TT	+	TT	-
	b	+		+		-		+		-	α	+		+		+
	c	-		-		+		-		+	a	-		-		-
	d	-		-		-		+		-	α	+		-		+
2 3	a	+	TT	-	NPD	-	PD	+	PD	-	α	+	NPD	-	TT	+
	b	+		+		+		-		+	a	-		+		-
	c	-		-		-		+		-	α	+		-		-
	d	+		+		+		-		+	a	-		+		+

Data Analysis

Spore viability. Tetrads that displayed more than two gene conversions were removed from the data. Error bars on spore viability are 95% confidence intervals determined from the Handbook of Biological Statistics (<http://udel.edu/~mcdonald/statintro.html>). Statistical differences between strains were determined with $2 \times 5 \chi^2$ with an $\alpha = 0.05$

Map distance. Map distances are expressed as centimorgans (cM) and were determined from Stahl Lab Online Tools (<http://www.molbio.uoregon.edu/~fstahl/>). This website uses the Papazian equation and gives map distances with standard error and significance between strains. This standard error is used as error bars on the map distances graph.

Interference. Interference values are given as ratios of map distances in CO:NCO fashion. For each interval, tetrads were separated based on the presence of a crossover in the adjacent interval. For example, in the URA3-HIS4 interval, tetrads were separated into two categories of “CO” (a crossover in the HIS4-LEU2 interval) or “NCO” (no crossover in the HIS4-LEU2 interval). Map distances were calculated for these two categories. This gives a map distance for

the URA3-HIS4 interval when a crossover was present in the HIS4-LEU2 interval and a map distance for the URA3-HIS4 interval when no crossover was present in the HIS4-LEU2 interval. These two map distances were compared using the CO:NCO ratio. A ratio of one shows no interference: the presence of a crossover in the HIS4-LEU2 interval does not influence the formation of a crossover in the URA3-HIS4 interval. A ratio less than one shows interference: the presence of a crossover in the HIS4-LEU2 interval decreases the likelihood of a crossover forming in the URA3-HIS4 interval. A ratio greater than one shows negative interference: the presence of a crossover in the HIS4-LEU2 interval increases the likelihood of a crossover forming in the URA3-HIS4 interval. Stahl Lab Online Tools were used to determine the map distances and statistical significance. This website uses the Perkins equation to determine map distances and variance. It uses a one-tailed and a two-tailed T test to determine significance between samples. This analysis was performed for all intervals in relation to the adjacent intervals on both sides of the analyzed interval.

E_0 Tetrads that showed parental ditypes in all intervals were determined to be E_0 's. Because of the possibility of a double crossover occurring with the same strands of DNA and showing itself as a parental ditype (false PD's), we needed to correct for these. Two crossovers in the same interval will result in 1PD:2TT:1NPD, depending on the strands used for the crossovers. So, PD's would happen as often as NPD's. This allows us to assume the number of false PD's is equal to the number of NPD's. So, for each interval, NPD's were subtracted from PD's to get the number of true PD's. This was then used to determine the probability of getting a false PD in each interval. The probability of false PD's in each interval was then summed to get the probability of False E_0 tetrads. This probability was then multiplied by the observed E_0 to get the

number of false E_0 's. False E_0 's were subtracted from observed E_0 's. A 2x2 Fisher's exact test was used to determine significant differences of percent E_0 between strains.

Gene conversion. Gene conversion is the name given to non-Mendelian segregation patterns.

Gene conversion occurs as a result of double strand breaks repairing off of the homolog instead of the sister chromatid. They can be accompanied by a crossover or a noncrossover. In this study we saw gene conversion manifest as a 3:1 or a 1:3 ratio of gene segregation, in contrast to 2:2 (Mendelian) gene segregation. Tetrads were assumed to be false tetrads and discarded if they showed gene conversions at more than two markers.

Assay of Crossover Assurance

Spore viability in Y55 strains. To determine if crossover assurance is functional in *tid1* mutants, chromosome III in the Y55 strain background was marked with genetic markers along its length (Figure 15). These markers were used to determine the location of crossovers on that chromosome. If crossover assurance is functional in the *tid1* mutant but not in the *ndj1* mutant, then the *ndj1* mutant would have more chromosomes lacking crossovers (E_0) than the *tid1* mutant. However, if crossover assurance is nonfunctional in both mutants then we expect to see a similar incidence of E_0 in both strains.

The *ndj1* mutant showed a decreased level of 4 spore viable tetrads and a high level of 3 and 2 spore viable tetrads, compared to wild type. The *tid1* mutant showed a large decrease in 4 spore viable tetrads than wild type and the *ndj1* mutant, and an increase in 3, 2, and 1 spore viable tetrads compared to wild type. The spore viability patterns of the *ndj1* and *tid1* mutants were significantly different from wild type and each other (Figure 20, Table 11, $2 \times 5 \chi^2 p < 0.001$). The *ndj1* mutant showed less of an increase in 0 spore viable tetrads, and more 3 spore viable tetrads than in the SK1 background, however these differences can be attributed to the different strain backgrounds.

Table 11. Y55 spore viability

Strain	No. Tetrads	Tetrads (%)				
		4 s.v.	3 s.v.	2 s.v.	1 s.v.	0 s.v.
WT	531	455	27	9	6	35
	(%)	85.5	5.1	1.7	1.1	6.6
<i>ndj1</i>	794	459	125	119	38	53
	(%)	57.8	15.7	15	4.8	6.7
<i>tid1</i>	1287	412	308	274	144	75
	(%)	32.1	23.9	21.3	11.2	11.5

Figure 20. Percent spore viability of Y55 native strains. Values are shown in table 11. Error bars are 95% confidence intervals.

Crossover Assurance is defective in *ndj1* and *tid1* mutants. Crossover assurance was assayed by determining the incidence of E_0 's of chromosome III. Heterozygous genetic markers placed along the length of a chromosome allowed detection of all crossovers between a homologous pair (Figure 15). This information can then be used to determine the number of crossovers formed along the length of chromosome III.

A significant increase in percent of E_0 was seen in both mutants compared to wild type (Table 12, $p < 0.001$). The percentages seen in the two mutants were not significantly different from each other. These results indicate no difference in crossover assurance between the two mutants.

Table 12. Incidence of E_0

	Corrected E _o Percentage	n
WT	1.3	399
<i>ndj1</i>	6.8 ($p < 0.001$)	386
<i>tid1</i>	8.4 ($p < 0.001$)	348

- p values reported are differences from wild type (Fisher's 2x2).
- E_o in *ndj1* and *tid1* are not significantly different from each other

Crossover assurance is defective in *ndj1* and *tid1* mutants. Map length in each interval was used as a measure of the amount of crossing over in that interval. Figure 21 and Table 13 show the map lengths of each interval for each strain. The only differences seen were in the *tid1* mutant in the KAN-HIS4 and HIS4-LEU2 intervals. The URA3-KAN^R interval had a small map length, indicating a low number of crossovers. This interval was not very useful to our measure of interference, and was therefore combined with the KAN^R-HIS4 interval to make the URA3-HIS4 interval.

Figure 21. Map lengths of each interval. * = significant difference from Wild Type, ** = significant difference between *ndj1* and *tid1* (Stahl Lab Online Tools).

Table 13. Map Distances with standard error.

Map Distance (cM)							
	URA-KAN	KAN-HIS	HIS-LEU	LEU-MAT	MAT-LYS	LYS-HYG	Total
WT	8.5 ± 1.3	24.9 ± 1.8	17.2 ± 1.3	29.5 ± 2.6	50.2 ± 2.9	18.9 ± 1.3	149
<i>ndj1</i>	7.9 ± 1.2	24.2 ± 1.9	18.1 ± 2.1	33.8 ± 2.6	52.9 ± 3.6	21.8 ± 1.9	159
<i>tid1</i>	5.6 ± 1.0	19.2 ± 1.9	11.2 ± 1.3	32.2 ± 2.7	44.9 ± 3.4	21.2 ± 2.0	134

S.E. are shown as ± values (Stahl Lab Online Tools).

Table 14. Raw data for intervals on chromosome III.

Interval	Strain	PD	TT	NPD	cM
URA-KAN	WT	282	51	2	8.5
	<i>ndj1</i>	281	36	0	7.9
	<i>tid1</i>	351	40	2	5.6
KAN-HIS	WT	181	141	4	24.9
	<i>ndj1</i>	176	128	3	24.2
	<i>tid1</i>	258	125	4	19.2
HIS-LEU	WT	217	112	1	17.2
	<i>ndj1</i>	216	84	5	18.1
	<i>tid1</i>	305	79	1	11.2
LEU-MAT	WT	190	144	11	29.5
	<i>ndj1</i>	155	154	12	33.8
	<i>tid1</i>	202	187	12	32.2
MAT-LYS	WT	84	238	21	50.2
	<i>ndj1</i>	104	192	24	52.9
	<i>tid1</i>	143	212	21	44.9
LYS-HYG	WT	208	132	1	18.9
	<i>ndj1</i>	201	112	4	21.8
	<i>tid1</i>	237	129	5	21.2

Our data allowed us to determine the extent to which crossover interference is functional in the three strains. Figure 22 and Table 15 show interference in each interval. Interference values are given as ratios of map distances in CO:NCO fashion. For each interval, tetrads were separated based on the presence of a crossover in the adjacent interval. A map distance was calculated for each condition and compared as the map distance for an interval when a crossover occurred in the adjacent interval to the map distance for an interval when no crossover occurred in the adjacent interval (See Materials and Methods section for further explanation). A ratio of one shows no interference, a ratio less than one shows positive interference, and a ratio greater than one shows negative interference.

The wild type showed a CO:NCO ratio of less than one in all intervals tested. Interference was stronger in the intervals around the HIS4 marker than any other interval in the wild type strain. The *ndj1* mutant showed significantly less interference than wild type (a CO:NCO ratio closer to 1) for most intervals (Two tailed T test). The *tid1* mutant only showed significantly less interference than wild type for two intervals (Two tailed T test), however all *tid1* mutant ratios were closer to one than wild type. Our data does show a trend towards defective interference in both the *ndj1* and *tid1* mutants, consistent with previously published data.

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Figure 22. CO:NCO ratios in each interval. This indicates the effect of a crossover in an adjacent interval on the interval tested. WT = blue, *ndj1* = red, *tid1* = green. Error bars are standard error (Stahl Lab Online Tools). * = Significant difference from Wild type (Two tailed T test, Stahl Lab Online Tools). There were no significant differences between *ndj1* and *tid1*.

Table 15. CO:NCO values for all intervals.

Affected interval	Strain	Affecting interval				
		URA-HIS	HIS-LEU	LEU-MAT	MAT-LYS	LYS-HYG
URA-HIS	WT		$0.26 \pm 0.06 *$			
	<i>ndj1</i>		$0.67 \pm 0.13 *+$			
	<i>tid1</i>		$0.56 \pm .14 *$			
HIS-LEU	WT	$0.22 \pm 0.04 *$		$0.70 \pm 0.11*$		
	<i>ndj1</i>	$0.78 \pm 0.19 +$		1.27 ± 0.29		
	<i>tid1</i>	$0.86 \pm 0.23 +$		1.05 ± 0.24		
LEU-MAT	WT	0.72 ± 0.13	$0.68 \pm 0.14*$		0.71 ± 0.14	0.98 ± 0.18
	<i>ndj1</i>	0.86 ± 0.14	1.04 ± 0.18		1.08 ± 0.19	0.86 ± 0.14
	<i>tid1</i>	$1.22 \pm 0.21 +$	0.94 ± 0.2		0.94 ± 0.17	1.36 ± 0.24
MAT-LYS	WT			$0.73 \pm 0.09 *$		0.80 ± 0.1
	<i>ndj1</i>			$1.19 \pm 0.17 +$		1.09 ± 0.15
	<i>tid1</i>			0.88 ± 0.13		1.12 ± 0.18
LYS-HYG	WT				0.80 ± 0.11	
	<i>ndj1</i>				$1.36 \pm 0.26 +$	
	<i>tid1</i>				1.04 ± 0.21	
<ul style="list-style-type: none"> • Positive interference is marked with an * (Stahl Lab Online Tools) • Significant differences from WT are Marked with + (Two tailed T test; Stahl Lab Online Tools) 						

Gene conversion. Figure 23 shows gene conversion at each marker. The only significant difference seen between strains was an increase in gene conversion in the *ycl076c::LYS2* marker in the *tid1* mutant ($p < 0.01$). Excluding the *LYS2* marker, our results were consistent with previously published data showing there was no significant difference in gene conversion between either the *tid1* or *ndj1* mutant and the WT strain (Shinohara et al. 2003; Chua and Roeder 1997).

Figure 23. Total gene conversion events at each marker. * = Significant difference from WT, ** = Significant difference from *ndj1* (Fisher's exact). There were 63 gene conversion events in WT, 82 in the *ndj1* mutant, and 73 in the *tid1* mutant.

Discussion

Crossover assurance and crossover interference seem to be manifestations of the same controls. Mutations of most ZMM proteins caused defects in both assurance and interference. However, the newly characterized ZMM protein, Spo16 shows a clear separation of the two mechanisms (Shinohara et al. 2008). Most mutants that have a defective crossover assurance mechanism show similar spore viability patterns: high 2 and 0 spore viable tetrads, indicative of meiosis I nondisjunction. Mutations in *TID1* showed a clear defect in crossover interference, however, did not show a spore viability pattern suggestive of a defect in crossover assurance (Shinohara et al. 2003). It seemed as though *tid1* mutants showed a separation of function mutation in which interference was disrupted, however assurance was left functional.

We set out to determine if a mutation in *TID1* disrupted crossover assurance. The SK1 *ndj1* and *tid1* mutants showed the same patterns in spore viability as the published data (Chua and Roeder 1997; Shinohara et al. 2003). The *ndj1* mutant showed a spore viability indicative of meiosis I nondisjunction, while the *tid1* mutant did not. Spore viability in the Y55 strain background was similar. The differences seen between the strains can be attributed to differences in strain backgrounds.

To determine if distributive disjunction is functional in the *tid1* mutant, we assayed the ability of wild type, *ndj1* and *tid1* mutants to segregate achiasmate chromosomes. Jon's thesis showed that *ndj1* homeologs in both Y55 and S288C had a spore viability pattern indicative of meiosis I nondisjunction and an inability to segregate the homeologous achiasmate chromosome (Figure 12 and 13). The S288C *tid1* homeolog, however, showed a pattern similar to the wild type (Figure 13) and significantly different from the S288C *ndj1* homeolog ($2 \times 5 \chi^2 p < 0.001$), indicative of a more normal meiosis I disjunction. These results indicate that the *tid1* homeolog retains some ability to segregate achiasmate chromosomes, suggesting an intact distributive

disjunction mechanism. This ability to segregate achiasmates may only be possible to an extent, and a large number of achiasmates may overwhelm the distributive disjunction mechanism. Since there was a forced achiasmate in chromosome III, any other achiasmates made because of the *tid1* mutation may not be segregated normally, possibly accounting for some of the spore death seen.

To determine if crossover assurance was functional in the *tid1* mutant, we assayed crossovers in intervals along the length of chromosome III in native Y55 yeast. As a direct readout of crossover assurance, we determined the incidence of E_0 chromosome III's. The *ndj1* and *tid1* mutant strains both had significantly higher incidences of E_0 than wild type (Fisher's 2×2 $p < 0.001$). This suggests that both mutants are equally defective in crossover assurance. This is interesting because this conclusion cannot be drawn from the spore viability pattern of the *tid1* mutant (Figure 11 and 20). The *tid1* spore viability pattern is suggestive of a functional crossover assurance mechanism because there is no increase in 0 spore viable tetrads compared to wild type.

Mutations in *NDJ1* and *TID1* have been previously shown to decrease crossover interference. Our data set was not robust enough to make any definitive conclusions, however we do show evidence that supports those previous findings. Interference was shown in most wild type intervals. For the *ndj1* and *tid1* mutants, crossover interference was decreased in all intervals, however was only significantly different from wild type in a few intervals (See Table 15). The absence of significance can be largely attributed to the sample size of our data set.

Our results along with previous data suggest a role for the Ndj1-dependent telomere tethering in both crossover assurance and crossover interference. Our results suggest that Ndj1 may also play a role in distributive disjunction, the cell's back-up mechanism. We also show that Tid1 is required for crossover assurance, but not for the distributive disjunction mechanism. We

fail to show evidence for a separation of function mutation in the *tid1* strain, further supporting the idea that crossover assurance and crossover interference are mechanistically linked.

Mutations in *NDJ1* result in defects in crossover assurance, crossover interference and distributive disjunction. This may be because of the structural role *Ndj1* plays in meiosis. It does not directly aid in double strand break repair, but plays a role in the physical location of the homologs in the nucleus. Any defects in the positioning of the homologs would not only decrease the ability of those homologs to form crossovers, but also hinder the ability of the homologs to become properly attached to the spindles.

Conversely, mutations in *TID1* result in defective crossover assurance and crossover interference, but do not affect distributive disjunction. This finding may be a function of the cellular role that *Tid1* plays in meiosis. *Tid1* is functionally involved in double strand break repair and plays no roles in chromatin placement within the nucleus. If the sole function of *Tid1* is to aid in double strand break repair, there would be no expectation of a defect in the back up mechanism of homolog segregation, distributive disjunction. It is possible that distributive disjunction requires that the homologs become attached to the spindles, but not necessarily physical connection of the homologs. This, of course, is not a very efficient method of segregating chromosomes because of the high possibility for nondisjunction of achiasmate chromosomes. However, one or two achiasmate chromosomes may be segregated properly as long as the homologs can align properly.

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